IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No.	: 09/936,173
Applicant	: Gai-Li JIAO et al.
Filed	: January 18, 2002.
TC/A.U.	: 1638
Examiner	: Georgia L. HELMER
Docket No.	: 2577-207
Customer No.	: 06449
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Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

DECLARATION UNDER 37 C.F.R. ' 1.132

I, Hong Yan, declare as follows:

1. I am a Principal Investigator of the Plant Biotechnology research group at Temasek Life Sciences Laboratory Limited, the assignee of the above-identified application. It is my understanding that I have been asked to provide this declaration because the inventors' whereabouts are currently not know.

2. My educational background and experience are shown in my *curriculum vitae* attached hereto as Exhibit A.

3. I have read and understand the Office Action mailed April 12, 2006, in which the Examiner has maintained a rejection of claims 4-8, 10, 11, 13-26, 28 and 30-36, which rejection was previously made in Office Actions mailed May 6, 2004 and September 9, 2005. It is the Examiner=s position that the application does not teach the broad applicability of transformation of cotton plants using different *Agrobacterium tumefaciens* strains and different marker genes. I disagree.

4. The following experiments were performed following the protocols described in the instant application. It is my understanding that these experiments were conducted by the inventors or under their supervision and control.

5. Fibrous root explants of cotton strain Coker 312 were transformed as described in the present application using *Agrobacterium* strain AGL1 containing the binary vector pPZP-GFP. The pPZP-GFP vector was derived from parent vector pPZP111, which is described by Hajdukiewicz et al. ("The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation." *Plant Mol Biol.* 1994 Sep;25(6):989-94.) Figure A of Exhibit B, attached hereto, shows a diagram of part of the vector pPZP-GFP, which contains expression cassettes for the GFP and NPTII marker genes. Fifteen transgenic cotton lines were obtained from these transformed fibrous root explants using the techniques described in the present application. The results of the analysis of these transgenic cotton lines are set forth in Exhibit B. One region of the GFP coding sequence and one region of the NPTII coding sequence shown by the black lines in Figure A were amplified and labeled as probes for genomic southern blot hybridization. The results are shown in Figures B and C. Figure B shows the results of genomic southern blot hybridization to the GFP probe of the 15 transgenic cotton lines. The lane with "M" is the DIG labeled lambda DNA digested by HindIII. Figure C shows the results of genomic southern blot hybridization to the NPTII probe of the 15 transgenic cotton lines. Figures B and C establish the 15 transgenic cotton lines contain the GFP marker and the NPTII marker in their genomes.

6. This data shows that all fifteen transgenic cotton lines were successfully transformed with the pPZP-GFP vector. This data further shows that cotton fibrous root explants were successfully transformed with the marker genes GFP and NTPII and that transgenic cotton plants were obtained using the techniques described in the present application.

7. Furthermore, all fifteen cotton plants were transformed using the *Agrobacterium* strain AGL1, establishing that transformation as performed according to the present application is not restricted to a given strain, but instead can be accomplished by a variety of strains.

8. One of ordinary skill in the art would be fully aware that the choice of vector and *Agrobacterium* strain is only limited by the requirement that different selectable markers are

present in the vector and in the *Agrobacterium* strain as indicated in, for example, Hajdukiewicz et al. Thus, one of skill in the art has broad flexibility in the choice of markers that they can use to successfully transform the fibrous root explants and broad flexibility in the choice of *Agrobacterium* strains that they can use to convey those markers into the fibrous root explants as taught by the present application.

9. In the Office Action mailed September 9, 2005, I note that the Examiner makes reference to the first line of page 10 of the specification. I believe that the Examiner has misread this sentence to be referring to a dependence of transformation on varieties of *Agrobacterium* strain. In fact, that sentence refers to the strain of cotton, not *Agrobacterium* and the difficulties in question are overcome by the advances represented by the present application as stated further on page 10.

10. In conclusion, the experiments described herein confirm that transformation of fibrous root explants performed as described in the application is a robust and adaptable procedure facilitating transformation by a broad range of marker genes and using a variety of *Agrobacterium* strains and that the application fully enables a person of skill in the art to practice the scope of the claimed subject matter.

11. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. All statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

2/11/06
Date

Yan HONG

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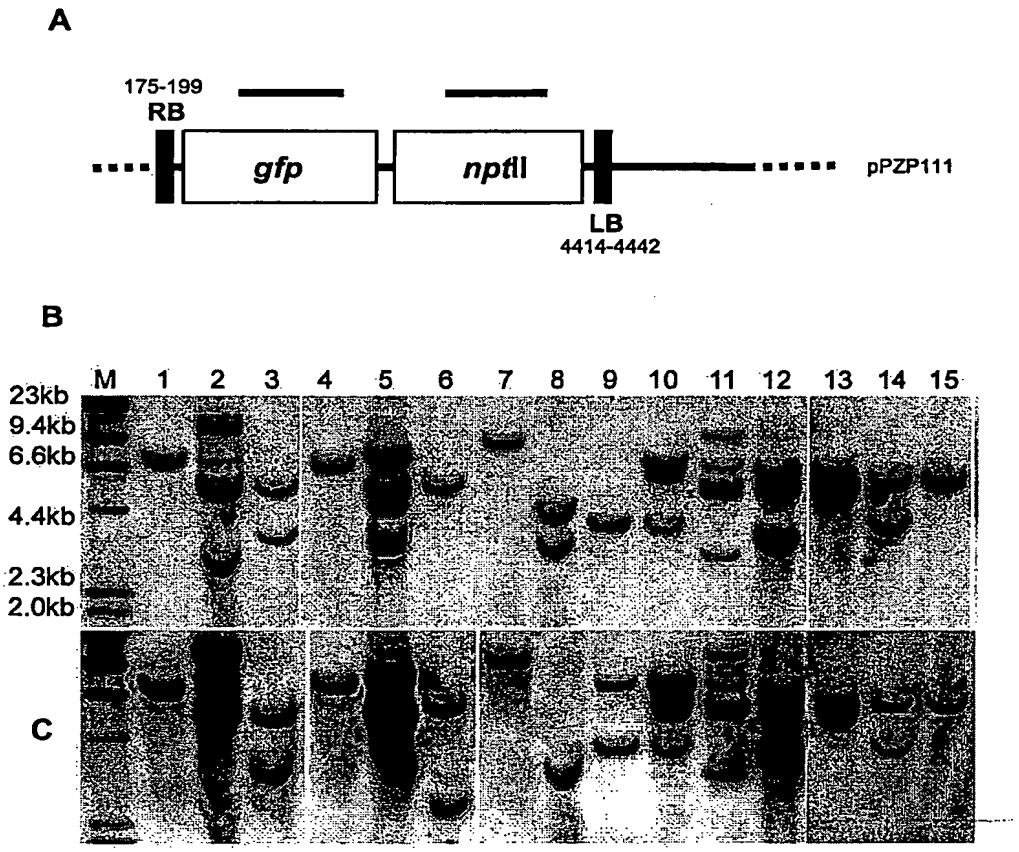

Molecular characterization of transgenic cotton lines derived from fibrous root explants infected by agrobacterium AGL1 with binary vector pPZP-GFP

A: The binary vector pPZP-GFP, which was derived by cloning a GFP encoding sequence into the *PstI*/*EcoR*I sites of the binary vector pPZP111 (Haidukiewicz et al. 1995). *npt*II and *gfp* are two expression cassettes in T-DNA encoding GFP protein and NPTII. One region for GFP coding sequence and one region for NPTII coding sequence (black lines) were amplified and labeled as probes for genomic southern blot hybridization.
B. Genomic southern blot hybridization to GFP probe of 15 transgenic cotton lines derived from fibrous root explant of Coker 312. M is the DIG labeled lamda DNA digested by HindIII.
C. Genomic southern blot hybridization to NPTII probe of the same 15 transgenic cotton lines.

Reference:
1. Hajdukiewicz, P., Svab, Z. & Maliga, P. *Plant Mol Biol* **25**, 989-94 (1994).

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Citizenship: Singapore

Education:

1984	BS	Sichuan University
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Employment history:

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1992-1995	Postdoctoral Research Fellow, Institute of Molecular and Cell Biology, Singapore
1995-1998	Acting Principle Investigator, Institute of Molecular Agrobiology
1998-2002	Biotechnology Research Manager, Institute of Molecular Agrobiology
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Other appointments:

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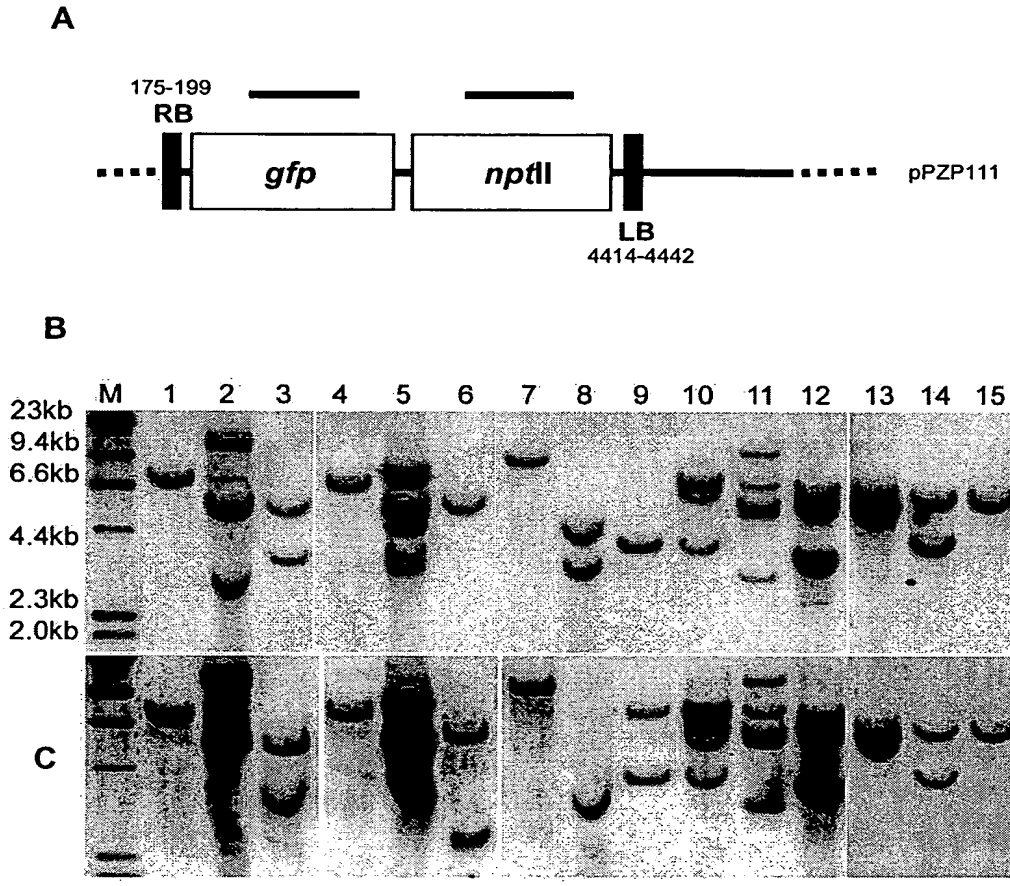
- Member, Genetic Modification Advisory Committee (GMAC), Singapore, subcommittee of agricultural products
- External Evaluator for Centre of Drug Administration (CDA), Singapore
- Reviewer for Biomedical Research Council (Singapore) grant application
- Reviewer for journals PLANT CELL REPORT, JOURNAL OF PLANT FUNCTIONAL GENOMICS, PLANT MOLECULAR BIOLOGY, NUCLEIC ACIDS RESEARCH, BMC BIOINFORMATICS

Recent Publications:

1. Bowman G.D., Nodelman I.M., **Hong Y.**, Chua N.H., Lindberg U. and Schutt C.E. (2000) A comparative Structural Analysis of the ADF/Cofilin Family. Proteins, Structure, function, and Genetics 41:374-384
2. Xie D.Y. and **Hong Y**. (2001) Regeneration of Acacia mangium through Somatic Embryogenesis. Plant Cell Reports 20:34-40
3. Dong C.H., Xia G.X., **Hong Y.**, Ramachandran S., Kost B. and Chua N.H. (2001) ADF Proteins are Involved in the Control of Flowering and Regulate F-actin Organization, Cell Expansion and Organ Growth in Arabidopsis. Plant Cell 13:1333-1346
4. Xie D.Y. and **Hong Y**. (2001) In vitro regeneration of Acacia Mangium via organogenesis. Plant Cell, tissue and Organ Culture 66:167-173
5. Xie D.Y. and **Hong Y** (2001) Agrobacterium-mediated genetic transformation of Acacia Mangium. Plant Cell Reports 20:917-922
6. Wang G.Y., Yuan M.F. and **Hong Y** (2002) In vitro Flower Induction of Roses. In Vitro Cellular and Developmental Biology-Plant 38:513-518
7. **Hong Y**. and A. Chuah. 2003. A format for databasing and comparison of AFLP fingerprint profile. BMC Bioinformatics 4:7

8. **Hong Y**. Gene discovery and characterisation for crop improvement. (2003) In *Agrobiotechnology and Plant Tissue Culture*. S.S. Bhojwani and W.Y. Soh (eds). Science Publishers, Inc.
9. Xiang N., Lam-Chan L.T., **Hong Y**. (2003) Genetic Analysis of Tropical Orchid Hybrids (*Dendrobium*) with Fluorescence Amplified Fragment Length Polymorphism (AFLP) J Am Soc Hort Sci128(5):731-735
10. Yuan M.F. and **Hong Y**. (2003) Heterogeneity of Chinese Medical Herbs in Singapore Assessed by Fluorescence Amplified Fragment Length Polymorphism (AFLP) Am J Chinese Med 31(5):773-779
11. Wang X.J., Cao X.L. and **Hong Y**. Isolation and characterisation of flower specific transcripts of Acacia mangium. (2005) Tree Physiology 25:167-178
12. Li S.J., Deng X.M., Mao H.Z., **Hong Y**. Enhanced anthocyanin synthesis in foliage plant Caladium Bicolor. (2005) Plant Cell Reports 23:716-720
13. Hong Y.Q., Lau A.J., Yeo C.L., Liu X.K., Yang C.R., Koh H.L., **Hong Y**. Genetic diversity and variation of saponin contents in *Panax notoginseng* roots from a single Farm. (2005) J Agri Food Chem 53:8460-8467
14. Zou P., **Hong Y.**, Koh H.L. (2005) Chemical fingerprinting of Isatis indigotica root by RP-HPLC and hierarchical clustering analysis. J Pharm Biomed Anal 38: 514-20
15. **Hong Y.** and Bhatnagar S. (2006) Tropical tree legumes. In: Biotechnology in Agriculture and Forestry, Tropical Crops. Pua, Eng-Chong; Douglas, Carl J. (eds.) Springer Verlag, Heidelberg, Germany (in press)
16. **Hong Y**. and Guo BL. (2006) DNA profiling for quality control of medical herbs, utilities and issues. In Current Review of Chinese Medicine-Quality control of herbs and herbal materials. Leung P.C., Fong H. and Xue C.C. (Eds.) World Scientific
17. **Hong Y**., Xiang N. and Lam-Chan L.T. (2006) Genetic Profiling for Identification of Plant Varieties Based on UPOV Principles: Fluorescent AFLP Analysis of *Dendrobium* Hybrids as an Example. in Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues (1st Edition), Teixeira da Silva JA (ed), Global Science Books. London, UK
18. Yue G.H., L .T. Lam-Chan and **Hong Y**. (2006) Development of simple sequence repeat (SSR) markers and their use in identification of *Dendrobium* varieties. Mol Ecology Notes 6:832-834

19. Lim A., Lim-Ho C.L., Yue.G.H. and **Hong Y**. (2006) Development and characterization of microsatellites in *Vanda* varieties. Mol Ecology Notes (in press)
20. **Hong Y**. (2006) Forestry Biotechnology is taking root. Asia Pacific Biotech News 10:302-304
21. **Hong Y**. (2006) DNA fingerprinting for botanicals. Innovation 6(2):36-37



Molecular characterization of transgenic cotton lines derived from fibrous root explants infected by agrobacterium AGL1 with binary vector pPZP-GFP

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1. Hajdukiewicz, P., Svab, Z. & Maliga, P. *Plant Mol Biol* **25**, 989-94 (1994).

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Plant Biology

Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium*

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Contributed by Mary-Dell Chilton, April 26, 1994

ABSTRACT **Inducible virulence (*vir*) genes of the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid are under control of a two-component regulatory system. In response to environmental factors (phenolic compounds, sugars, pH) VirA protein phosphorylates VirG, which in turn interacts with the promoters of other *vir* genes, causing induction. A mutation of *virG*, *virG*N54D (which codes for a Asn-54 → Asp amino acid change in the product), causes constitutive expression of other *vir* genes independent of *virA*. We have investigated whether providing *Agrobacterium* with a plasmid containing *virG*N54D augments the efficiency of transfer of the T-DNA (transferred DNA). For both tobacco and cotton, we observed an enhancement of transformation efficiency when the inciting *Agrobacterium* strain carries the *virG*N54D mutation. We also tested whether supplying *Agrobacterium* with a similar plasmid containing wild-type *virG* affects the efficiency of T-DNA transfer. An intermediate efficiency was observed when this plasmid was employed. Using a β-glucuronidase (GUS) reporter gene to assess transient expression of T-DNA after transfer to tobacco and maize tissues, we observed a higher frequency of GUS-expressing foci after inoculation with *Agrobacterium* strains carrying *virG*N54D than with *Agrobacterium* carrying the wild-type *virG*. Gene-transfer efficiency to maize by an octopine strain was greatly improved upon introduction of *virG*N54D. Multiple copies of wild-type *virG* were equally effective in promoting transient expression efficiency in tobacco but were virtually ineffective in maize. We propose the use of *virG*N54D to improve the efficiency of *Agrobacterium*-mediated transformation, especially for recalcitrant plant species.**

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* incite crown gall tumors and hairy root disease, respectively, on a wide range of dicotyledonous plants and gymnosperms and on some monocotyledonous plants. Virulent strains of *Agrobacterium* harbor a large tumor-inducing (Ti) or root-inducing (Ri) plasmid. During the process of infection at wound sites in susceptible plants, a specific segment of the plasmid, T-DNA (transferred DNA), is transferred from the bacterium to the host plant cells and is inserted into the nuclear genome. Ti plasmid T-DNA contains genes for synthesis of cytokinin and auxin, phytohormones that cause proliferation of plant cells to form the gall (reviewed in ref. 1). The transformed plant cells are also directed by T-DNA genes to produce unusual metabolites called opines, such as octopine and nopaline, that serve as specific nutrients for the inciting bacteria (2) and are often used to type Ti plasmids.

T-DNA on the Ti plasmid is bordered by directly repeated 25-bp sequences that are required in cis for its excision and transfer. No other part of T-DNA is necessary for its transfer (3). Excision and transfer functions of T-DNA are mediated by virulence functions that map on the Ti plasmid outside T-DNA and are encoded by several *vir* genes and operons (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*). Expression of the virulence genes results in the production in *Agrobacterium* of one or more linear copies of the lower strand of the T-DNA called "T-strands" (4, 5). The T-strand is capped at the 5′ end by the *virD2* gene product (6, 7) and is protected by a single-strand binding protein encoded by *virE2* (8, 9). The T-strand is believed to be directed to the plant nucleus by nuclear localization sequences in VirD2 and VirE2 proteins (10–12). T-DNA integrates into the plant chromosomes at seemingly random locations by an unknown mechanism.

Virulence functions are transcriptionally regulated by a two-component gene-regulatory system that responds to the chemical environment (13). Optimal *vir* gene induction occurs at acidic pH and in the presence of phenolic inducers, such as acetosyringone (AS) (14), that are released by wounded plant cells. The *vir* gene regulatory system operates through two monocistronic virulence genes: *virA* and *virG*. The constitutively expressed *virA* gene produces a protein located in the inner membrane that recognizes plant wound metabolites (3). The resulting autophosphorylation of VirA protein activates the intracellular signal-transducing protein VirG by phosphorylation of an aspartic acid residue (15). The activated VirG binds to *vir* gene promoters at conserved *vir* box sequences and acts as a transcriptional activator of the virulence genes (16, 17).

Many dicot plants and gymnosperms are susceptible to *Agrobacterium* and are potentially transformable by *Agrobacterium*, although at various efficiencies. Most monocots such as maize appear to be less susceptible to *Agrobacterium* infection. However, synthesis of opines has been reported for young maize seedlings after *Agrobacterium* inoculation (18). Expression of a chimeric β-glucuronidase (GUS) gene carried by the T-DNA was also reported to be expressed in excised maize seedling apices (19) and in shoots of young maize seedlings (20). Moreover, T-DNA transfer from *Agrobacterium* to maize cells has been demonstrated by agroinfection, a technique using *Agrobacterium*-mediated transfer of viral sequences to plant cells (21).

There may be several problematic steps that can limit the efficiency of transformation, but surely one of the most critical steps is the induction of the virulence genes. In the case of maize, which is not efficiently transformable by *Agrobacterium*, it has been shown that 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), a metabolite found in maize homogenates, specifically inhibits the induction of Ti plasmid virulence genes at sublethal concentration (22). Expression of *vir* genes independent of environmental parameters could allow *Agrobacterium* to transfer T-DNA more efficiently to monocot plants such as maize. It has been shown that an increase in copy number of *virA* and

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Abbreviations: AS, acetosyringone; GUS, β-glucuronidase; MS, Murashige and Skoog medium; T-DNA, transferred DNA; Ti and Ri plasmids, tumor-inducing and root-inducing plasmids; Km, kanamycin; NptII, neomycin phosphotransferase II; DIMBOA, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one.

†To whom reprint requests should be addressed.

virG genes can partially relieve the pH dependence of *vir* gene induction (23, 24). Recently, we have isolated a mutant of *virG*, *virG*N54D (with an altered codon that codes for a Asn-54 → Asp amino acid change in the product), that allows high-level constitutive *vir* gene expression in the absence of *virA* and chemical inducing agents (25). We present here an assessment of the effect of *virG*N54D on the efficiency of *Agrobacterium*-mediated transformation of tobacco and cotton and on the frequency of transient expression in tobacco and maize.

MATERIALS AND METHODS

Strains and Plasmids. Plasmid pAD1288 was constructed by cloning a blunt-ended 5-kb *Eco*RI–*Hin*dIII restriction fragment containing pRiHRI origin of replication (26) into the blunt-ended *Hin*dIII site of pGP109 (25). pGP109 harbors a copy of the *virG* gene from the octopine Ti plasmid pTiA6. pAD1289 is the corresponding plasmid carrying the mutant *virG*N54D. The binary vector pBG5 is a pBIN19 derivative that contains a *nos*-Npt II-*nos* selectable marker gene for plant transformation conferring kanamycin (Km) resistance (neomycin phosphotransferase II gene bracketed by the promoter and terminator of the nopaline synthase gene *nos*) and a 35S-GUS-*nos* gene coding for GUS under the control of the cauliflower mosaic virus 35S promoter and the *nos* terminator (20). This construct shows no detectable expression in *Agrobacterium* (20). Further details of the strains used in this study are listed in Table 1.

Media. Bacteria were cultured in YEB medium (5 g of beef extract, 1 g of yeast extract, 5 g of Bactopeptone, and 5 g of sucrose per liter, pH 7.2) with the following concentrations of selective antibiotics as appropriate for the various plasmids: ampicillin at 100 mg/liter (pAD1288 and pAD1289) or Km at 100 mg/liter (pBG5). Plant tissues were cultivated on Murashige–Skoog medium (MS) (28) supplemented with Km at 25 or 100 mg/liter and cefotaxime at 500 mg/liter where indicated.

Plant Growth and Inoculation Conditions. *Infection of tobacco.* Transformation of *Nicotiana tabacum* cv. Xanthi was carried out by leaf disc inoculation (29). Tobacco leaf discs from 4- to 5-week-old axenically grown tobacco plants were immersed in *Agrobacterium* suspension (10^9 cells per ml) for 15 min and plated on MS medium. After cocultivation for 2 days, leaf discs were transferred to fresh MS medium supplemented with 100 mg of Km and 500 mg of cefotaxime per liter and incubated at 25°C with a 16-hr photoperiod.

Infection of cotton. Seeds of *Gossypium hirsutum* L. cv Deltapine 50 were surface-sterilized with 50% Clorox for 20 min, rinsed three times in sterile water, and germinated on MS medium. Infection of cotton was performed as described (30). Cotyledons of 12-day-old cotton seedlings were cut into 1-cm^2 pieces and immersed in an *Agrobacterium* suspension (10^9 cells per ml) for 10 min. The pieces were then placed on callus initiation medium (30). Tissues were incubated at 25°C with a 16-hr photoperiod. After 3 days of cocultivation, the cotyledon tissues were placed on the same medium containing 25 mg of Km and 500 mg of cefotaxime per liter and incubated at 30°C with a 16-hr photoperiod.

Infection of maize shoots. The infection of maize shoots was performed essentially as described (20). Immature zygotic embryos (14 days after pollination; *ca.* 2 mm long) of the maize line A188 were picked from greenhouse-grown ears. Excised embryos were plated on MS medium with 3% sucrose and incubated at 25°C under a 16-hr photoperiod. Shoots were excised from 3-day-old seedlings by cutting at the coleoptilar node. Shoots were immersed in an *Agrobacterium* suspension (10^9 cells per ml), subjected to vacuum infiltration for 10 min, and then cultured on MS medium supplemented with 100 μM AS for 3 days at 25°C under a 16-hr photoperiod. Shoots were stained for 2 days with 0.052% 5-bromo-4-chloro-3-indolyl glucuronide in 100 mM NaH_2PO_4 (pH 7). Shoots were then destained for 1 day with ethanol to remove chlorophyll before scoring blue spots.

Infection of tobacco seedlings. An overnight culture (5 ml) of *Agrobacterium* grown in YEB medium was washed and resuspended in 10 ml of MS medium. Axenic seed-grown tobacco plantlets of *Nicotiana tabacum* cv. Xanthi, grown for 12 days in a growth chamber at 26°C with a 16-hr photoperiod, were immersed in the bacterial suspension and subjected to vacuum infiltration for 15 min (12). The plantlets were then placed on MS plates. After cocultivation for 3 days in a growth chamber at 26°C, the plantlets were analyzed by histochemical GUS stain (31). The plantlets were destained with ethanol to remove chlorophyll before scoring blue spots.

Table 1. *Agrobacterium* strains used in this study

Strains	Chromosomal background	Ti plasmid
C58	C58	pTiC58 (a nopaline-type plasmid)
Ach5	Ach5	pTiAch5 (an octopine-type plasmid)
LBA4404	Ach5	pAL4404 (a deletion mutant of pTiAch5 lacking T-DNA but having a full set of *vir* genes; ref. 27)

RESULTS

Stable Transformation of Tobacco and Cotton. The effect of *virG*N54D on plant transformation was investigated by assaying transformation efficiency on tobacco and on cotton. Supplementary copies of *virG* or *virG*N54D were introduced into the desired *Agrobacterium* strain by introducing pAD1288 or pAD1289, respectively. All strains in addition contained plasmid pBG5 (20), a plasmid carrying a modified 35S-GUS-*nos* gene and the *nos*-NptII-*nos* gene as a plant selectable marker between T-DNA borders. After inoculation with these strains, plant tissues were plated on hormone-free medium containing Km to allow growth of transformed tissues only—i.e., cells that acquired hormone independence from integration of the T-DNA of pTiC58 or pTiAch5 as well as Km resistance from the expression of the NptII gene of pBG5. Transformation efficiency was measured by quantification of Km-resistant hormone-independent calli.

The transformation efficiency of *Agrobacterium* strains C58 and Ach5 containing the various constructs was assayed on tobacco leaf discs and cotton cotyledon tissues. Experiments were performed on discs of equal size, and inoculations were done with equal concentrations of bacterial cells to compare transformation efficiency. The number of individual Km-resistant tumors was scored 2 weeks after inoculation for tobacco and 4 weeks after inoculation for cotton. Table 2 summarizes the results of three independent experiments.

Patterns for transformation of tobacco leaf discs and cotton cotyledons were similar. In harmony with previous studies (32), we observed that both the octopine and the nopaline strains of *Agrobacterium* with pBG5 and pAD1288 carrying the wild-type *virG* exhibited a 2- to 3-fold increase in transformation efficiency compared with the same strain with only pBG5. *Agrobacterium* with pAD1289 carrying *virG*N54D exhibited a further increase in transformation efficiency (Table 2). Both tobacco and cotton could be transformed almost quantitatively (70–93%) with either the octopine or the nopaline strain containing *virG*N54D. Transformation efficiency of strains with the control *virG* construct (pAD1288) was 50–65%.

Table 2. Effect of additional copies of *virG* (pAD1288) and of *virG*N54D (pAD1289) on transformation of tobacco leaf discs and cotton cotyledon segments

Strains	No. of leaf discs with Km-resistant tumors/total no. tested (%)					
	Tobacco			Cotton		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
C58 (pBG5)	16/46 (35)	15/41 (36)	12/30 (40)	4/20 (20)	3/18 (16)	4/22 (18)
C58 (pBG5, pAD1288)	47/72 (65)	48/80 (60)	50/80 (62)	21/40 (54)	19/38 (50)	19/35 (54)
C58 (pBG5, pAD1289)	61/71 (86)	68/80 (85)	71/80 (88)	31/40 (77)	28/40 (70)	30/35 (85)
Ach5 (pBG5)	10/30 (33)	12/30 (40)	12/30 (40)	5/19 (26)	4/20 (20)	3/20 (15)
Ach5 (pBG5, pAD1288)	43/70 (61)	38/60 (63)	50/80 (62)	25/40 (62)	25/40 (62)	22/40 (55)
Ach5 (pBG5, pAD1289)	61/70 (87)	56/60 (93)	71/80 (88)	35/40 (87)	34/40 (82)	28/40 (70)

We noted an additional effect of providing *Agrobacterium* with the *virG*- or *virG*N54D-containing plasmid: tumors appeared at an earlier time, and the number of individual tumors per disc increased to 8–10 for tobacco and 2–3 for cotton. With the parental strains C58(pBG5) and Ach5(pBG5), only 2 or 3 tumors per disc formed on tobacco and 1 or 2 on cotton cotyledons.

Strains of *Agrobacterium* without the plasmid pBG5 were also used to infect cotton and tobacco tissues as described above. Infected tissues were plated on MS with or without Km. The number of tumors on MS medium observed 2 or 4 weeks after inoculation for tobacco tissues and cotton tissues, respectively, matched the frequency of Km-resistant tumors in the corresponding pBG5-containing strain (data not shown). As expected, usually no tumors were detected on MS with Km; in rare cases, however, small tumors emerged but did not show further growth (data not shown).

Transient Expression in Maize Shoots. We investigated the effect of *virG* and *virG*N54D on transient expression efficiency in young maize and tobacco seedlings. Maize was chosen because it is relatively refractory to *Agrobacterium* infection, although both transient expression (20, 21) and stable transformation (18, 19) of maize by *Agrobacterium* have been reported. In this study, the T-DNA transfer assay relied upon expression of a chimeric GUS gene in plant tissues 3 days after inoculation. All of the strains of *Agrobacterium* tested contained a plasmid with a 35S-GUS gene (pBG5) (20) to allow monitoring of the transient expression of transferred genes in these plant tissues.

To determine the frequency of transient transformation of maize tissues, we used the protocol of Shen *et al.* (20). Shoots excised from 3-day-old seedlings grown from immature embryos of the maize line A188 were infected with *Agrobacterium*, and histochemical GUS assays were performed 3 days after infection. AS was included in the cocultivation agar, but bacteria were not preinduced. It has been shown that pretreatment of dicot plant tissues with AS (33) or use of AS during cocultivation (34) improved transformation efficiency by enhancing plant cell division (33).

Results of these studies, summarized in Table 3, revealed interesting differences between the octopine and nopaline strains. In nopaline strain C58(pBG5), blue spots indicating GUS activity were observed on 36–40% of the shoots after cocultivation. The effect of adding plasmid pAD1288 (with *virG*) appeared nil (35–42%); however, the addition of pAD1289 (with *virG*N54D) to this strain led to a 2-fold increase in the percentage of shoots showing blue spots (78–83%). The impact of *virG*N54D was even more dramatic when the octopine strain LBA4404 was used for infection. As reported previously (20), the octopine strain LBA4404 showed a very low efficiency of DNA transfer (0–15% of shoots with blue spots). A slight increase in DNA transfer efficiency (10–25%) was observed when plasmid pAD1288 (with *virG*) was introduced into this strain. Strikingly, when pAD1289 containing *virG*N54D was introduced into this strain, 70–77% of the shoots showed GUS-positive blue spots, indicating an efficiency of DNA transfer similar to that of the nopaline strain in the presence of *virG*N54D.

Transient Expression in Tobacco. In transient expression assays with tobacco, both pAD1288 and pAD1289 led to a similar increase (2–4 fold) in DNA transfer efficiency (Table 3). C58(pBG5) produced GUS-positive blue spots in only 16–25% of tobacco seedlings. Addition of pAD1288 carrying *virG* increased this frequency to 66–75%; and addition of pAD1289 carrying *virG*N54D produced 75–83% frequency of transiently expressing plantlets. Strain LBA4404 (pBG5) produced GUS-positive blue spots 33–50% of the time. Addition of the plasmid carrying *virG* increased this value to 83%, while that with *virG*N54D gave similar results (83–91%). However, the number of GUS-positive blue spots was higher when the infecting bacteria contained *virG*N54D strain (Fig. 1).

To assure that these GUS-positive spots are indeed the consequence of transfer of T-DNA into the plant cells, we used as control a T-DNA transfer-deficient *Agrobacterium* strain containing an insertion mutation in *virB*. Under the experimental conditions used in our study, tobacco seedlings and maize shoots inoculated with this strain did not exhibit any blue spots (data not shown).

DISCUSSION

***virG*N54D Improves Stable Transformation Efficiency.** In this study, plasmid pAD1289 carrying *virG*N54D in the

Table 3. Transient GUS expression on shoots of maize seedlings and on tobacco plantlets after cocultivation with *Agrobacterium* and its mutants

Strains	No. of tissues stained blue/total no. tested (%)				
	Maize			Tobacco	
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2
C58 (pBG5)	18/50 (36)	21/54 (38)	16/40 (40)	2/12 (16)	3/12 (25)
C58 (pBG5, pAD1288)	15/42 (35)	19/48 (39)	17/40 (42)	8/12 (66)	9/12 (75)
C58 (pBG5, pAD1289)	20/24 (83)	25/32 (78)	24/30 (78)	9/12 (75)	10/12 (83)
LBA4404 (pBG5)	3/20 (15)	2/18 (11)	0/21 (0)	4/12 (33)	6/12 (50)
LBA4404 (pBG5, pAD1288)	2/20 (10)	5/20 (25)	3/18 (16)	10/12 (83)	10/12 (83)
LBA4404 (pBG5, pAD1289)	36/48 (77)	15/21 (71)	28/40 (70)	10/12 (83)	11/12 (91)

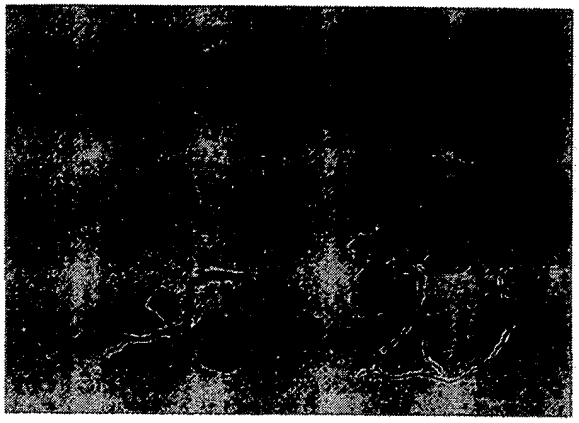

FIG. 1. Tobacco seedlings inoculated with LBA4404(pBG5) (*Lower Left*) and LBA4404(pBG5) containing pAD1288 (*Upper Center*) or pAD1289 (*Lower Right*). Plant tissues were stained with 5-bromo-4-chloro-3-indolyl glucuronide 3 days after inoculation. The discs were photographed after soaking for 48 hr in 70% ethanol.

infecting strain enhanced the efficiency of *Agrobacterium*-mediated transformation in both tobacco and cotton. *Agrobacterium* containing this mutant *virG* exhibits constitutive high levels of *vir* gene expression in the absence of *virA* and plant phenolic inducers (24, 25). Infection of tobacco leaf discs with a strain containing *virG*N54D resulted in a 2-fold increase in frequency of transformed leaf discs compared with the parental strain. In cotton cotyledon transformation, an increase of *ca.* 4-fold was observed. When plasmid pAD1288 containing wild-type *virG* was introduced into *Agrobacterium* as a control, an intermediate frequency of transformation was observed (1.5-fold increase for tobacco, 3-fold for cotton). These results indicate that induction of *vir* genes is indeed a limiting feature of tumor induction in these systems and that *virG*N54D can confer superior T-DNA transfer efficiency.

***virG*N54D Renders Octopine-Type *Agrobacterium* Highly Infectious on Maize.** In maize, octopine-type *Agrobacterium* strains are much less efficient than nopaline-type strains in agroinfection (21, 35) and in producing transient expression of T-DNA (20). The inefficiency could be corrected in large part by addition of a plasmid containing the *virA* gene from the nopaline-type plasmid (36). The results reported here show that addition of *virG*N54D circumvents the problem of the octopine strain in transient infection of maize, rendering it about as effective as the nopaline strain. This result is fully consistent with the interpretation (reviewed in ref. 37) that *vir* gene induction is indeed the problematic step for maize infection by the octopine strains.

***virG* Effects on Efficiency of Transient Expression.** Liu *et al.* (32) have demonstrated that addition of a multicopy plasmid carrying *virG* to an *Agrobacterium* strain enhanced the transient transformation frequency for celery, carrot, and rice tissues. Our studies further explore the scope of this effect. In tobacco, the frequency of plants expressing GUS transiently was significantly increased by addition of pAD1288 carrying wild-type *virG* to the infecting strain. However, we find that provision of this plasmid to either C58- or LBA4404-derived *Agrobacterium* strains produces little or no increase in the frequency of transient expression in maize. One significant methodological difference is that Liu *et al.* (32) preinduced the *Agrobacterium* with AS for 24 hr before infection of plant tissues. In this study, bacteria were not preinduced, but AS was included in the cocultivation agar. It is plausible that preinduction of *vir* genes with AS would be especially important for maize seedling infection because DIMBOA produced by maize can presumably inhibit induction of *vir* genes during bacterial invasion of the plant (22).

Role of *virG* in Hypervirulence. The key role of *virG* in determining transformation efficiency is clear from the finding that hypervirulent *Agrobacterium* Ti plasmid pTiBo542 has a normally virulent T-DNA (38) but a hypervirulent form of *virG* (39). The hypervirulence of pTiBo542 *virG* is mediated by higher levels of expression of *vir* genes after induction (40). Under optimal induction conditions, the coding region (which has two amino acid substitutions) is responsible for the hypervirulence of this gene. The constitutive *virG*N54D used in this study has a single amino acid substitution that maps between the pTiBo542 *virG* changes (39). Although a constitutive mutant version of this supervirulent *virG* might be expected to produce superior constitutive levels of *vir* gene expression, a mutation that converts Asn-54 to Asp in the pTiBo542 *virG* gene produced a slightly lower level of *virB:lacZ* expression than does the pTiA6 *virG*N54D (24).

Implications for Vector Design. Disarmed Ti/Ri plasmids or binary vectors have been used to transfer genes for herbicide resistance, disease resistance, and insect resistance into economically important crops (reviewed in ref. 41). In *Agrobacterium* strains with *virG*N54D, T-DNA transfer can occur immediately and thus should be less subject to adverse environmental factors. This should be especially important in recalcitrant plants, which may lack sufficient or appropriate inducer chemicals and/or, like maize (22), may produce secondary metabolites that are inhibitors of induction. Recalcitrant plants may also produce toxic compounds that reduce the *Agrobacterium* population before the bacteria can mount an induction and transfer T-DNA. Constitutive *vir* gene expression conferred by *virG*N54D should enable *Agrobacterium* to transfer T-DNA immediately with no lead time for induction. We propose that incorporation of *virG*N54D into transformation vectors outside T-DNA will improve the efficiency of *Agrobacterium*-mediated transformation. We further suggest that this stratagem may be particularly valuable for plants that appear refractory or even immune to *Agrobacterium* T-DNA transfer.

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1. Kado, C. I. (1993) in *Bacterial Conjugation*, ed. Clewell, D. B. (Plenum, New York), pp. 243–254.
2. Dessaux, Y., Petit, A. & Tempé, J. (1991) in *Molecular Signals in Plant Microbe Communications*, ed. Verma, D. P. S. (CRC, Boca Raton, FL), pp. 109–136.
3. Zambryski, P. C. (1992) *Annu. Rev. Plant Physiol.* **43**, 465–490.
4. Stachel, S. E., Timmermann, B. & Zambryski, P. (1986) *Nature (London)* **322**, 706–712.
5. Albright, L. M., Yanofsky, M. F., Leroux, B., Ma, D. & Nester, E. W. (1987) *J. Bacteriol.* **169**, 1046–1055.
6. Ward, E. R. & Barnes, W. M. (1988) *Science* **242**, 927–930.
7. Young, M. F. & Nester, E. W. (1988) *J. Bacteriol.* **170**, 3367–3374.
8. Gielt, C., Koukolíková-Nicola, Z. & Hohn, B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9006–9010.
9. Christie, P. J., Ward, J. E., Winans, S. C. & Nester, E. W. (1988) *J. Bacteriol.* **170**, 2659–2667.
10. Herrera-Estrella, A., Van Montagu, M. & Wang, K. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9534–9537.
11. Citovsky, V., Zupan, J., Warnick, D. & Zambryski, P. (1992) *Science* **256**, 1803–1805.
12. Rossi, L., Hohn, B. & Tinland, B. (1993) *Mol. Gen. Genet.* **239**, 345–353.
13. Leroux, B., Yanofsky, M., Winans, W., Ward, J., Ziegler, S. & Nester, E. (1987) *EMBO J.* **6**, 849–856.
14. Stachel, S. & Zambryski, P. (1986) *Cell* **46**, 325–333.

15. Jin, S., Prusti, R., Roitsch, T., Ankenbauer, R. & Nester, E. W. (1990) *J. Bacteriol.* **172**, 4945–4950.
16. Jin, S., Roitsch, T., Christie, P. & Nester, E. W. (1990) *J. Bacteriol.* **171**, 2506–2512.
17. Pazour, G. J. & Das, A. (1990) *J. Bacteriol.* **172**, 1241–1249.
18. Graves, A. C. F. & Goldman, S. L. (1986) *Plant Mol. Biol.* **7**, 43–50.
19. Gould, J., Devey, M., Hasegawa, O., Ulian, E. C., Peterson, G. & Smith, R. H. (1991) *Plant Physiol.* **95**, 426–434.
20. Shen, W.-H., Escudero, J., Schläppi, M., Ramos, C., Hohn, B. & Koukolíková-Nicola, Z. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1488–1492.
21. Grimsley, N., Hohn, T., Davies, J. W. & Hohn, B. (1987) *Nature (London)* **325**, 177–179.
22. Sahi, S. V., Chilton, M.-D. & Chilton, W. S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3879–3883.
23. Pazour, G. P. & Das, A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6941–6945.
24. Jin, S., Pan, S. Q. & Nester, E. W. (1993) *Mol. Microbiol.* **7**, 555–562.
25. Pazour, G. P., Ta, C. N. & Das, A. (1992) *J. Bacteriol.* **174**, 4169–4174.
26. Jouanin, L., Vilaine, F., d'Enfert, C. & Casse-Delbart, F. (1985) *Mol. Gen. Genet.* **201**, 370–374.
27. Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. & Schilperoort, R. A. (1983) *Nature (London)* **303**, 179–180.
28. Murashige, T. & Skoog, F. (1962) *Physiol. Plant* **15**, 473–497.
29. Horsch, R., Rogers, S. & Fraley, R. T. (1985) *Cold Spring Harbor Symp. Quant. Biol.* **50**, 433–437.
30. Firoozabady, E., DeBoer, D. L., Merlo, D. J., Halk, E. L., Amerson, L. N., Rashka, K. E. & Murray, E. E. (1987) *Plant Mol. Biol.* **10**, 105–116.
31. Jefferson, R. A. (1987) *Plant Mol. Biol. Rep.* **5**, 387–405.
32. Liu, C.-N., Li, X.-Q. & Gelvin, S. B. (1992) *Plant Mol. Biol.* **20**, 1071–1087.
33. Guivarc'h, A., Caissard, J.-C., Brown, S., Marie, D., Dewitte, W., Van Onckelen, H. & Chriqui, D. (1993) *Protoplasma* **174**, 10–18.
34. Owens, L. D. & Smigocki, A. C. (1988) *Plant Physiol.* **88**, 570–573.
35. Boulton, M. I., Buchholz, W. G., Marks, M. S., Markham, P. G. & Davies, J. W. (1989) *Plant Mol. Biol.* **12**, 31–40.
36. Raineri, D. M., Boulton, M. I., Davies, J. W. & Nester, E. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3549–3553.
37. Chilton, M.-D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3119–3120.
38. Hood, E. E., Helmer, G. L., Fraley, R. T. & Chilton, M.-D. (1986) *J. Bacteriol.* **168**, 1291–1301.
39. Chen, C.-Y., Wang, L. & Winans, S. C. (1991) *Mol. Gen. Genet.* **230**, 302–309.
40. Jin, S., Komari, T., Gordon, M. P. & Nester, E. W. (1987) *J. Bacteriol.* **169**, 4417–4425.
41. Gasser, C. S. & Fraley, R. T. (1989) *Science* **244**, 1293–1299.

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Abstract

Somatic embryogenesis and regeneration of whole plants is a highly genotype-dependent process in cotton. We have identified at least one highly regenerable Australian cultivar, Siokra 1-3, which is a sister line to the current major variety being grown in Australia. A number of plants have been regenerated and although some are showing abnormal pollen development, most can produce fertile seed when selfed or crossed with a normal pollen donor.

Agrobacterium tumefaciens has been used to efficiently produce fertile transgenic Siokra 1-3 plants expressing novel genes such as the bacterial neomycin phosphotransferase or the β-glucuronidase. This is the first example of the transformation of an elite commercial cultivar. Critical factors in the transformation are the use of a supervirulent disarmed Ti-plasmid with a binary transformation vector, and a highly regenerable genotype of cotton. Bacterial concentration at the time of infection, tissue age, kanamycin selection regime, and co-cultivation support and media composition all have an influence on transformation efficiency and were optimised in our protocol.

The ability to transform an elite Australian cultivar of cotton paves the way for agronomic improvements through genetic engineering. We have concentrated on increasing the tolerance of Australian cotton to the herbicide 2,4-D (to protect it from spray drift damage from adjacent cereal crops), and increasing its tolerance to insect pests, such as *Helicoverpa armigera*, using BT-toxin genes, protease inhibitors and other novel insect resistance genes.

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Publish by Abstract

Adaptation of Cotton Shoot Apex Culture to *Agrobacterium*-Mediated Transformation

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Abstract. A protocol is presented for rapid genotype-independent transformation and regeneration of cotton (*Gossypium* spp.) from shoots isolated from germinating seedlings. Isolated shoots are inoculated with a super-virulent strain of *Agrobacterium tumefaciens*, subjected to a mild antibiotic selection, and directly regenerated as shoots *in vitro*. Shoots do not dedifferentiate and mutation rates are low. Rooted shoots can be obtained within 6–10 weeks of isolation and inoculation depending on the cotton cultivar.

Key words: *Agrobacterium*, cotton, *Gossypium*, shoot apex, transformation

Introduction

The*Agrobacterium*-mediated transformation method described here was developed from the genotype-independent shoot apex plant regeneration method we originated for cotton (Gould and Smith, 1988; Gould et al., 1991a). This transformation and regeneration approach (Smith et al., 1992) has been used in *Agrobacterium*-mediated transformation of petunia (Ulian et al., 1988), maize (Gould et al., 1991b) and rice (Park et al., 1997). A more efficient and detailed procedure is described here. Changes to earlier procedures include use of precultured shoots in the inoculation step to insure activation of cell division in apical meristematic tissues, and inclusion of cytokinin in the culture medium during selection to promote shoot growth.

Use of the *Agrobacterium* vector is technically simple and gene transfers are often low copy, permanent and heritable. In this method, genetic transformation occurs following inoculation of dividing meristematic cells in a shoot apex with a supervirulent strain of *A. tumefaciens*. Hormonal manipulation is kept to a minimum to permit the native developmental program

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in the apex to control plant regeneration, allowing regeneration to be plant-driven and, therefore, genotype-independent. Plants generate directly from inoculated shoots on a simple MS-based medium (Murashige and Skoog; 1962; Shabde and Murashige, 1977) that has been used to regenerate many cultivars of *Gossypium* (Gould et al., 1991a), maize (Gould et al., 1991b) and other cereals. No other hormonal manipulations are necessary unless shoot multiplication is desired (Hemphill et al., 1998).

Shoot meristem and apex cultures became popular in the ornamental nursery industry after the discovery that rapidly growing shoots of many virus-infected clones could be free of virus and used to produce virus-free germplasm (Morel and Martin, 1952). Over time, it was observed that the incidence of genetic mutations and somaclonal variation was low in plants regenerated from shoots. One of the reasons for this low mutation frequency may be the absence of tissue dedifferentiation steps that are common in the initiation of callus and somatic embryo cultures. This event is known to trigger retrotransposon activity in cultured plant tissues and produces permanent mutations (Hirochika, 1993).

The procedure described here uses transformation-competent cells in the shoot apex of germinating seedlings for *Agrobacterium*-mediated transformation and for plant-driven regeneration. Mature seed is used because it is readily available and easily germinated when needed. Isolation and inoculation of shoots is followed by regeneration of normal, fertile plants that flower and set viable seed. An overall transformation efficiency of 5–10% or greater can be expected.

Materials and Methods

Plant material and culture media

Start with fresh, de-linted seed of any desired variety of cotton (*Gossypium* spp.). Culture medium is made from pre-mixed Murashige & Skoog, 1962 (MS) inorganic salt formulation and minimal organics that includes myo-inositol and thiamin (can be purchased through Sigma (M-9274), or Gibco BRL, Grand Island NY).

Seed germination medium (SG)

Eight grams of Bacto Agar in 1 liter of water, dispense SG into sterile petri-plates @ 25 ml/plate, or $\frac{1}{2}$ pint glass canning jars, or baby food jars at approx. 20 ml/jar. Cover jars with inverted petri dish.

Plant culture media (MS)

All plant media are based on the **MS** formulation (Murashige and Skoog, 1962) (M-9274 Sigma), solidified with 8 g/L agar, sterilized by autoclaving, and dispensed into sterile plastic petri dishes (deep dishes are best).

MS+Kin: MS + kinetin 0.1 mg/L (Gould et al., 1991a; Shabde and Murashige, 1977).

MS+C: MS+carbenicillin (Sigma) 500 mg/L (or Clavamox® 250 mg/L).

MS+Kin+C+Kan: MS+kinetin 0.1 mg/L, carbenicillin, 500 mg/L (or Clavamox®, 250 mg/L) + kanamycin 30–50 mg/L.

MS+AC: MS + 0.5–1% charcoal (activated and neutralized).

LB: Bacterial culture media is solidified using 15 g/L agar (Sambrook et al., 1989).

LB+Kan: LB + kanamycin, used with *A. tumefaciens* EHA105 strain and most other *Agrobacterium* vectors, except EHA101.

LB+Kan+X: LB + kanamycin, 50 mg/L + X (appropriate antibiotic), for the EHA101 strain (which has native chromosomal kanamycin resistance) containing a binary vector carrying resistance to a different specific antibiotic (X) (i.e., pGA482, tetracycline 5–10 mg/L).

Vir **IM**: *Vir* Introduction medium is seventy-five mM MES pH 5.4 (Wayne Barnes, personal communication), 2% glucose (Fullner et al., 1996), and acetosyringone 30–100 μM (Veluthambi et al., 1989). We use 50 μM acetosyringone, octopine 100 mg/L (Veluthambi et al., 1989; Raineri et al., 1990), Kinetin 100 mg/L.

Potting soil

2 portions peat: 1 washed river sand: 2 vermiculite, coarse grade (2:1:2). If 1 part = 1 cu. ft., then add: 500 ml dolomite, 250 mL gypsum and slow-release macro- and micronutrients (Courtesy of Joe Thaxton, USDA, Southern Crops Regional Facility). Cotton needs a neutral pH and is sensitive to calcium deficiency (use 1 or 2 cherry tomato plants as indicator plants for calcium deficiency).

Bacteria and culture media

A. tumefaciens, EHA101 (Hood et al., 1986), EHA105 or other super-virulent strain. Strike out a fresh plate of *Agrobacterium* two days prior to shoot inoculation. Grow bacteria for two days on agar-solidified **LB** medium containing selective antibiotic on desk or bench top, 20–27 °C. Do not exceed 28 °C.

A. tumefaciens is a soil bacterium and virulence can be lost when incubated at 28 °C or higher.

Virulence induction medium **IM** contains activators and protectors of the *Agrobacterium* virulence genes, which can increase transformation rate. After two days growth on **LB**, described above, scrape bacteria from one culture plate and mix with 1 ml *vir***IM** to make a thick bacterial suspension. Vortex briefly and use immediately.

Binary vectors

A binary vector carrying the desired genes must be transferred to *A. tumefaciens* (Mersereau et al., 1990). The binary vectors pGA482 (An, 1987) carrying tetracycline resistance, or pPZP 200 series (Hajdukiewicz et al., 1994) carrying spectinomycin resistance are suitable for use with EHA101. The binary vectors pGA482, pPZP 100 series, pBI101 series including pBI121, or pBIN19 can be used with EHA105.

Method

1. **Seed sterilization.** Cotton seed can be difficult to decontaminate and a number of approaches work; however, only one is presented here. Wrap de-linted seeds in cheesecloth and soak in water 30 min. Remove water and soak seeds in H_2O_2 (30%) for 30 min. Rinse 3X with sterilized water. Soak seeds in 50% Chlorox solution for 30 min, change the solution every 10 min. Remove Chlorox and rinse seeds 4X in sterilized water. Leave seeds in final rinse 30 min–1 h.
2. **Seed germination.** Squeeze seeds out of the seed coat and culture onto **SG** medium to germinate 7 days, in light, at 27–30 °C. Germination will take longer if the temperature is lower. Removing the seed coat prior to germination reduces contamination.
3. **Shoot isolation and preculture.** The seedling shoot is embedded in the stem between the cotyledons. Remove one cotyledon by pushing down on it until it snaps off (Figures 1 and 2). This exposes the shoot apex (described in Gould et al., 1991a). The shoot (epicotyl) is removed from the seedling and cultured in **MS+Kin** for 3–5 days, 5 shoots/plate. Remove shoots if they are contaminated. This step helps initiate cell division in the apex and allows identification of contaminated shoots prior to inoculation.
4. **Inoculation**. Two approaches to shoot inoculation are described. Alternative approaches can be used as long as bacteria are applied to meristem regions (Figure 3).

Table 1. Inoculation and schedule – cotton shoot apex transformation

Procedure	Time	Media and Components
Seed germination	5–7 days in light	Seed Germination Medium (**SG**)
Shoot isolation and pre-culture	3–7 days	**MS**(Sigma M-9274)+ 0.1 mg/l **kinetin (MS+kin)**
Agrobacterium culture	1–2 days 20–24 °C	EHA101(pTi) **(LB+Kan+Tet)** EHA105(pTi) **(LB+Kan)**
Shoot apex isolation and inoculation	$\frac{1}{2}$ day	Scrape new growth of bacteria from overnight culture. Mix bacteria with 1 ml ***vir* Induction Medium** (***vir* IM**): 75 mM MES pH=5.4; 100 uM, acetosyringone, 100 mg/L octopine, 2% glucose, 10mg/100ml kinetin.
Co-cultivation	3–7 days	**MS** + 0.1 mg/l **kinetin (MS+kin)**
Reculture	1 week	**MS + carbenicillin** 500 mg/l **(MS+C)**
Selection	1–2 weeks	**MS + carbenicillin** 500 mg/l+0.1 mg/l **kinetin + kanamycin** Test: 10–50 mg/l **(MS+C+K+Kin)**
	1–2 weeks	Selection can be repeated 1 week.
Rooting	1–4 weeks 2 weeks	**MS + Charcoal 3% (MS+AC)** If roots don't form, transfer to **MS+Kin** and allow shoots to enlarge. If shoots do not root, try options **a.** or **b.** **a.** Transfer large non-rooted shoots to soil following treatment of stem with rooting hormone powder (Rootone). Maintain high humidity and light. **b.** Graft non-rooted shoots onto germinated cotton seedlings.

I. Place shoot tissue shoot upright and 'shave' the shoot on two opposing sides creating a wedge of tissue with the shoot meristem in the center.

II. Shoots are bisected from apex to base producing two asymmetrical 'halves'. Inoculate and culture both halves although only the larger half will grow. Inoculate wound with *A. tumefaciens* mixed 1:1 in virulence induction medium (*vir* **IM**).

In both I and II, remove excess tissue from shoot base. Do not remove leaf primordia or elongating leaves, since these structures supply hormones and other growth factors that aid development of the shoot on the simple medium used (Shabde and Murashige, 1977). Place approximately 5 inoculated shoots onto 1 plate of **MS+Kin**.

5. **Co-cultivation (MS+Kin)**. Once all shoots have been prepared, inoculate shoot region generously with *Agrobacterium* mixed 1:1 with *vir* **IM**. Vacuum infiltrate 5 min. Keep the cultures in light on desk or bench top. Incubate 19–22 °C if possible to promote formation of bacterial pili (Fullner, 1996). Do not put the cultures under direct light, such as in a culture room, because the interior of the culture can become too hot. If condensation forms inside the culture plate, the culture is too hot. During cocultivation, check for bacterial overgrowth and move shoots to a clean spot on the plate if overgrowth occurs. Co-cultivate for 3–7 days. This amount of time is sufficient for genomic incorporation, transcription and accumulation of transcripts to occur, to aid survival during selection.
6. **Culture (MS+C)**. Transfer shoots to **MS+C** for 7 days. In **MS+C** and in **MS+Kin+C+Kan** we use Clavamox® (amoxicillin plus clavulanic acid, an inhibitor of penicillinase activity, Smith/Kline Bechamp, Veternary) in place of carbenicillin. It is relatively inexpensive and it is effective in killing *Agrobacteria* at low concentrations (250 mg/L), yet is not toxic to plant tissues at high concentrations (10,000 mg/L). It can be obtained through a veterinary pharmacy or a Veterinarian. Clavamox®, comes as sterile individually packaged tablets that are dissolved and suspended in 5 ml sterile water and added directly to cooled autoclaved media.
7. **Selection (MS+Kin+C+Kan)**. Transfer to **MS+Kin+C+Kan** selection medium containing 30–50 mg/L kanamycin and 500 mg/L carbenicillin (or Clavamox®) for 7 days to 2 weeks, at the rate of 5 shoots/plate. Small shoot explants of cotton are sensitive to kanamycin because of the prevalence of cell division. In this meristem-based method, if the meristem is killed, the procedure fails. One of the current problems in the shoot apex method is that the promoters used to drive *neo* (in many cases it is the *nos* promoter in *npt*II), may not be efficiently used in plant

meristem. Under these circumstances, the selection pressure used must be low and escapes occur; however, transformed shoots are not killed. If most shoots appear to be dying after 7 days to 2 weeks, transfer to rooting medium **MS+AC** (described below). However, if most shoots look robust after 7 days to 2 weeks of culture, reculture onto **MS+Kin+C +Kan** for an additional 2 weeks.

8. **Rooting (MS+AC).** Surviving shoots will root spontaneously on **MS+AC** in 2 to 4 weeks. Culture 3 shoots/plate. Shoots that do not root should be returned to **MS+Kin** for 2–4 weeks, then transferred to MS+AC. Allow shoots to grow and root. Remove rooted plants from agar, rinse well, treat with fungicide (Subdue+Benlyate) and transfer to hydrated Jiffy 7 peat pellet contained in a 1 pint canning jar or magenta box. Place under high light, cover with a lid from a plastic Petri plate and keep watered. Once the root system becomes established, leaves will enlarge and plants will begin to grow vigorously. If shoots haven't rooted, there are two options:

   I. Dip the shoot stem in rooting powder, transfer to hydrated Jiffy 7 pellets and maintain under relatively high light. Water rooted shoots, treat with fungicide and keep in a sealed zip-lock bag until shoot begins to grow.

   II. Another alternative is to graft the shoot onto a germinated seedling of the same variety (Luo and Gould, unpublished).

9. **Flowering and pollination.** Flowering of the R_0 plants occurs within 20 days depending on the amount of light the plants receive and the temperature. In the field, cotton is insect pollinated. Cotton plants grown in a greenhouse are self-pollinated. Plants may need to be hand pollinated to insure self-pollination when determining segregation patterns.

10. **Progeny analyses.** Collect seed (R_1 , R_2... generation) and delint. Greenhouse-grown seeds are relatively free of contaminating organisms, therefore, sterilization does not need to be intensive. Surface sterilize de-linted seeds with 20% fresh bleach for 25 min, rinse 3X in sterile water, and germinate in water/agar (**SG**), or Jiffy peat pellets. Screen seedlings using GUS activity (Jefferson, 1988), and PCR amplification for the transferred genes. Transfer to soil and to the greenhouse. Under greenhouse conditions, cotton is self-pollinated and the transferred genes should segregate in the R_1 in a 3:1 pattern (75% will be positive). If more copies have been transferred, the ratio of plants carrying the gene will be greater. If regenerated plants are chimeric, any pattern of transformation in the R_1 can be expected.

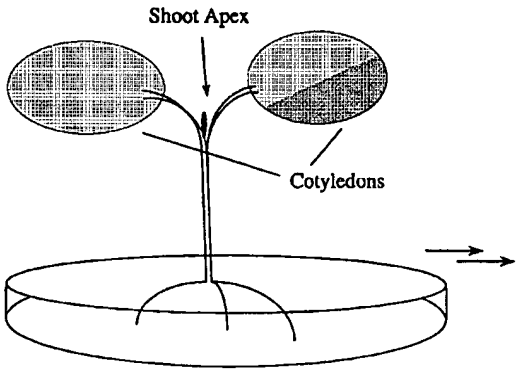

Figure 1. The seedling shoot is embedded in the stem between the cotyledons.

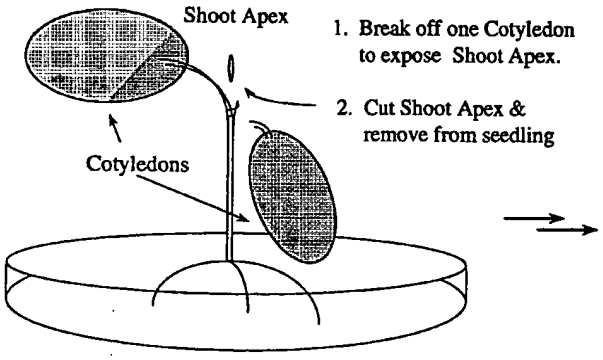

Figure 2. Remove one cotyledon by pushing down until it snaps off. This exposes the shoot apex. The seedling shoot apex, or epicotyl, is removed from the seedling and cultured in **MS+Kin** for 3–5 days.

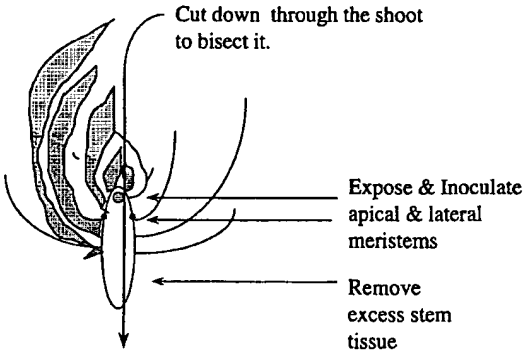

Figure 3. A lateral section of the cotton shoot is removed to expose the meristems in the apical region. This area is then inoculated with *Agrobacterium* and recultured.

Discussion

Three factors are important in *Agrobacterium* mediated transformation:

I. Presence of actively dividing cells (transformation-competent cells) in the plant apex.

II. Use of a 'super-virulent' strain of *A. tumefaciens* and induction of virulence.

III. An antibiotic selection level tailored to the level of promoter activity in the inoculated apical meristem and explant size, to avoid killing transformed tissues.

1. Transformation competent cells. In the procedure outlined here, isolated and inoculated seedling shoot apices are immediately cultured on **MS+Kin** to promote cell division in the apex and aid regeneration of the apical meristem. Inclusion of cytokinin in the culture medium helps to insure that some cells in the apex will be transformation competent.

2. Bacterial virulence. The super-virulent and broad host-range *A. tumefaciens* strains (EHA101, EHA105 and similar strains) are important for successful transformation of cotton shoots. Virulence can be induced by a variety of methods to insure activation of *vir* gene transcription. We have used *vir* **IM,** acetosyringone, nopaline or octopine in MES buffer (Veluthambi et al., 1989; Rainieri et al., 1990; Fuller et al., 1996; Wayne Barnes, personal communication).

3. Selection protocol. At this time, a permissive selection protocol is important in the practice of this method because plant regeneration is dependent on vitality of the meristem. The promoter used with the selectable resistance gene such as *neo*, must be active in the meristem for selection to work as one would normally expect. If cells in the meristem die, the organization inherent in the meristem is destroyed and regeneration from the meristem is abolished. In *nptII*, the *nos* promoter drives *neo*. If the *nos* promoter is used, protection to 10–30 mg/L of kanamycin can be expected from small, recently inoculated shoots. The CaMV 35S promoter is not active in plant meristem or shoot apex and if used with the antibiotic resistance gene, conferres *no* protection to this vital region. The amount of selection tolerated also depends on the size and preparation of the shoot explant, which is operator dependent and highly individualistic. Ten mg/L is recommended for the first selection passage. If too many escapes are produced following this procedure, increase the level of selection.

Note

Regenerated plants (R_0) should be considered chimeric. Upon analysis, transfered genes may be present in less than single copy, or present as multiple insertion events. For Southern blot

analysis, it is best to use the progeny (R_1, R_2, etc.) of self pollinated or outcrossed regenerated individuals. The transgenic progeny will not be chimeric and will reflect the transformation event that was present in the germ line.

References

An G, Watson BD, Stachel S, Gordon MP and Nester EW (1985) New cloning vehicles for transformation of higher plants. EMBO J 4: 277–284.

Fullner KJ, Lara, JC and Nester EW (1996) Pilus assembly by *Agrobacterium* T-DNA transfer genes. Science 273: 1107–1109.

Gould J and Smith R (1988) Shoot tip culture as a potential transformation system. Beltwide Cotton Production Conferences, National Cotton Council, Memphis TN. pp. 113–114.

Gould J, Banister S, Fahima M, Hasegawa O and Smith RH (1991a) Regeneration of *Gossypium hirsutum* and *G. barbadense* from the shoot apex. Plant Cell Rept 10: 12–16.

Gould J, Devey M, Hasegawa O, Ulian EC, Peterson G and Smith RH (1991b) Transformation of *Zea mays* L., using *Agrobacterium tumefaciens* and the shoot apex. Plant Physiol 95: 426–434.

Gould J, Zhou Y, Shen Y, Magallanes-Cedeno M and Luo J (1997) Shoot apex transformation of cotton using *Agrobacterium.* Beltwide Cotton Production Conferences, National Cotton Council, Memphis TN. pp. 432–434.

Hajdukiewicz P, Suab Z and Maliga P (1994) The small versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25: 989–994.

Hemphill JK, Maier CG and Chapman KD (1998) Rapid *in-vitro* plant regeneration of cotton (*Gossypium hirsutum* L.). Plant Cell Rept 17: 273–278.

Hirochika H (1993) Activation of tobacco retrotransposons during tissue culture. EMBO J 12: 2521–2528.

Hood E, Helmer R and Fraley RT (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTBo542 outside of T-DNA. J Bact 168: 1291–1301.

Hood E, Gelvin S, Melchers L and Hoekema A (1986) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2: 208–218.

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Reptr 5: 387–405.

Mersereau M, Pazour GJ and Das A (1990) Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene 90: 149–151.

Morel G and Martin C (1952) Guerison de dahlias atteints d'une maladie a virus. C.R. Seance Acad Sci, Paris 233: 1324–1325.

Murashige T and Skoog F (1962) A revised medium for rapid growth of and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497.

Shabde M and Murashige T (1977) Hormonal requirements of excised *Dianthus caryophyllus* L. shoot apical meristem *in vitro*. Am J Bot 64: 443–448.

Smith R, Gould J, Ulian E (1992) Method for transforming plants via the shoot apex. US Pat. 5,164,310.

Ulian EC, Smith RH, Gould J and McKnight T (1988) Transformation of plants via the shoot apex. *In Vitro* Cell Dev Biol 24: 951–954.

Veluthambi K, Krishnan M, Gould J, Smith RH and Gelvin S (1989) Opines stimulate induction of the vir genes of *Agrobacterium tumefaciens* Ti plasmid. J Bact 171: 3696–3703.

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